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Laser-patterned stem-cell bridges in a cardiac muscle model for on-chip electrical conductivity analyses

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Abstract

Following myocardial infarction there is an irreversible loss of cardiomyocytes that results in the alteration of electrical propagation in the heart. Restoration of functional electrical properties of the damaged heart muscle is essential to recover from the infarction. While there are a few reports that demonstrate that fibroblasts can form junctions that transmit electrical signals, a potential alternative using the injection of stem cells has emerged as a promising cellular therapy; however, stem-cell electrical conductivity within the cardiac muscle fiber is unknown. In this study, an *in vitro* cardiac muscle model was established on an MEA-based biochip with multiple cardiomyocytes that mimic cardiac tissue structure. Using a laser beam, stem cells were inserted adjacent to each muscle fiber (cell bridge model) and allowed to form cell-cell contact as determined by the formation of gap junctions. The electrical conductivity of stem cells was assessed and compared with the electrical conductivities of cardiomyocytes and fibroblasts. Results showed that stem cell-myocyte contacts exhibited higher and more stable conduction velocities than myocyte-fibroblast contacts, which indicated that stem cells have higher electrical compatibility with native cardiac muscle fibers than cardiac fibroblasts.

Introduction

Electrical conduction within the heart normally begins with generation of an action potential by a cardiac pacemaker cell, resulting in the depolarization of surrounding cells, and then initiation of a propagating wavefront and subsequent unimpeded propagation of depolarization in a cardiac tissue.¹ Some disease conditions such as ischemia or infarction can lead to changes in tissue structures, notably formation of nonconductive fibrous tissue. These nonfunctional regions can vary in size from single-cell interspersions to large blocks, leading to conduction disruption and blockage and subsequent arrhythmias.² Stem-cell transplantation aims at regenerating functional tissue within the damaged heart. This new therapeutic technique requires that conducting properties of the stem-cell graft must match

that of the host tissue to provide unimpeded conduction. Currently, *in vitro* studies on electrical conductivity of stem cells are performed in random cell-culture models,^{3,4} which cannot provide precise information relevant to the heart, in which muscle cells are aligned to form cardiac fibers. The electrical propagation in the heart, especially in the ventricles, is unidirectional along the aligned muscle fiber; this intrinsic unidirectional propagation prevents arrhythmias. In the random cell-culture model, in which cardiac cells were seeded in the culture dishes without any geometric controls, the electrical signals propagate in multiple directions in the cell monolayer.⁵ Therefore, a reproducible and *in vivo*-mimicking model is especially important for systematically investigating stem-cell electrical conductivity. In this study, an *in vivo*-like cardiac muscle was constructed as a one-dimensional conduction model to study stem-cell conductivity.

To rapidly assess electrical propagation along each cardiac muscle fiber, the cardiac muscle model was constructed on microelectrode arrays (MEAs). When an action potential was generated on the cell membrane, a spike waveform associated with the corresponding extracellular field potential could be measured by one electrode on the MEA. Only when the cell that generated the action potential was within 10 to 20 μm range relative to the electrode could the spike recorded by the electrode have sufficiently high magnitude; this provided a high spatial resolution for recording the locally activated electrical signals.⁶ Because the electrodes on the MEAs could record the extracellular field potential without damage to the cell membrane, long-term assessments on electrical conductivity from the same cell culture were possible. To date, study of MEA-based electrical conductivity was achieved by randomly culturing a large amount of cardiomyocytes over the MEA surface.⁷ Initially, two-dimensional models were developed by seeding cells on the MEA surface without any spatial restriction. Very weak control of cell orientation and arrangement in two-dimensional models made it difficult to perform unidirectional electrical conduction through the cell culture. The results were ambiguous for evaluating and comparing electrical conductivity on different culture samples or on the same sample on different culture days. Therefore, one-dimensional cardiac muscle models were established by restricting the cell culture in a narrow strip on the MEA as multiple muscle fibers. These cardiac muscle fibers permitted unidirectional electrical conduction along only a linear pathway, which allowed for a relatively high throughput and systematic assessment of electrical conductivity.

Cardiomyocytes in native myocardial tissue are organized in parallel to form cardiac muscle fibers. The intracellular contractile myofibrils are aligned along the fiber, and the intercellular junction proteins concentrate at the ends of the cardiomyocytes.⁸ This highly oriented cellular architecture is critical for proper electromechanical coupling between cardiomyocytes. However, in conventional cultures, cardiomyocytes spread with disorganized myofibrils and diffusive junctions, losing their normal myocardial morphology and functions.⁹ Therefore, based on the concept that structure determines function, it is essential to establish a culture model with *in vivo*-like morphology to restore and maintain the normal electrical function of cardiomyocytes. With such a model, stem-cell electrical conductivity can be assessed under an *in vivo*-like condition that is significant for cell transplantation into the heart tissue. Several methods have been successfully used in aligning cardiomyocytes to build *in vitro* cardiac muscle fibers. These methods include microcontact printing¹⁰ and microfluidics,¹¹ in which cell adhesion proteins (*e.g.*, fibronectin, laminin, or collagen I) are patterned onto the polystyrene cell culture dishes to guide cardiomyocyte alignment. With such approaches, however, it is difficult to transfer and immobilize the proteins onto the MEA surface. In this study, we used lithographic techniques to microfabricate a biochip with multiple microwells in which cardiomyocytes can be aligned to form *in vivo*-like cardiac muscle fibers under geometric restriction.

To study cell electrical conductivity based on a cardiac muscle model, cell bridges were created on each muscle fiber. The electrical signal propagation through the cell bridge was assessed by calculating the electrical conduction velocities of the cells that formed the bridge. Cell-bridge methods associated with cardiac muscle fiber models were developed in previous studies.¹² For example, a narrow spacer was used to selectively block a segment of the micropatterned fibronectin to prevent cell attachment while cells were seeded to form the muscle fiber. After the spacer was removed, various types of cells were seeded onto the blocked area to form a cell bridge. However, this method of randomly filling cells to form the cell bridge cannot control the number of cells placed for the bridge. In the study reported here, we used a micropipette to remove a defined portion of each cardiac muscle fiber and then used the laser-patterning technique¹³ to deposit a controlled number of cells of a selected cell type to form the cell bridge. Our goal was to create a defined cell bridge between two separated cardiomyocyte clusters aligned as an *in vivo*-like cardiac muscle fiber model to systematically study stem cell conductivity.

Materials and methods

Model design

The cardiac muscle model was established by creating microwells on MEAs. A microfabricated elastomeric membrane with eight through slots (1.5 mm in length, 30 μm in width, and 40 μm in height) was bonded onto the surface of an MEA chip. The chip contained 8×8 indium tin oxide (ITO) electrodes without TiN coating (electrode diameter and spacing were 30 μm and 200 μm , respectively). Each slot was aligned with one line of MEA electrodes to form a long microwell that contained eight electrodes as shown in Fig. 1(a). Cardiomyocytes were seeded onto the membrane immediately after cell harvest and grew inside the microwells to form eight cardiac muscle fibers. After the cardiomyocytes formed fiber morphology, a portion of each fiber was removed by a glass micropipette to form a gap.

The laser-patterning technique utilizes optical force, which can confine a single cell in the axis of a laser beam and guide it downward to a substrate.¹⁴ By moving the substrate relative to the laser beam, selected cells can be moved transversely while they are pushed down to create a specific cell pattern. Using this technique, individual stem cells were laser-patterned into the gap (created using the glass micropipette) to connect the two separated parts of the muscle fiber as shown in Fig. 1(b). Electrical activities were recorded along each cardiac muscle fiber, and local conduction velocity was calculated between two adjacent electrodes. Since the width of the fiber was smaller than the cardiac electrotonic space constant, electrical propagation along the fiber was pseudo-1D.¹⁵ This allowed microscopic tracking of electrical propagation along a distinct linear path without ambiguity about the exact pattern of electrical signal propagation. In this setting, stem cells were tested for their ability to bridge electrical conduction within a cardiac muscle fiber, and the results were compared with cardiomyocyte bridges (positive control) and fibroblast bridges (negative control).

Cell manipulation

Neonatal rat cardiomyocytes—Cardiomyocytes were isolated and collected from three-day-old neonatal rats using a two-day protocol. Ten neonatal rats were dissected, and the hearts were collected and minced in Moscona's Saline. The heart tissue was transferred into 50 mL Dulbecco's Phosphate Buffered Saline (DPBS) with 4 mg trypsin and 50 mg neutral protease and stored in a 4 °C refrigerator overnight. The next day, the heart tissue was transferred into 50 mL Krebs Ringers Bicarbonate Buffer (KRB) with 10 mg collagenase type I and 30 mg collagenase type II and then shaken in a water bath at 50 RPM for 1 h. The

cell suspension was washed twice using cardiomyocyte culture medium (high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% fetal bovine serum and 1% penicillin streptomycin) to remove the enzyme residue. The isolated cells were transferred into a 150 cm² flask to remove the cardiac fibroblasts through the cell adhesive assay. After two hours, the unattached cardiomyocytes were collected for further use. Our immunocyto staining data and data from other groups that used the same cardiomyocyte purification procedure have demonstrated that 95% cardiomyocyte purification can be obtained.

Rat mesenchymal stem cells from bone marrow (rMSCs-bm)—rMSCs, purchased from ScienCell™ research laboratories, were cryopreserved at passage-one culture and delivered frozen. They were characterized by the immunofluorescent method with antibodies to CD73, CD90, CD105, and Oil Red staining after adipodifferentiation. The rMSCs were cultured using mesenchymal stem-cell medium provided by the same company and used for coculture before the fifth passage. The rMSCs were labeled by DiI before laser-patterning to be distinguishable from cardiomyocytes in the coculture. The cardiomyocyte culture medium was used as the coculture medium.

All experiments that involve animal use were performed in compliance with the relevant laws and institutional guidelines. These experiments have been approved by Clemson University's Institutional Animal Care and Use Committee through protocol AUP2010-032.

Biochip construction

The biochip was created by bonding a microfabricated elastomeric membrane onto the surface of an MEA chip, which was used for on-chip analysis of electrical conductivity. Elastomeric membranes were microfabricated in polydimethylsiloxane (PDMS) using standard photolithography and soft lithography.¹⁶ The microscale features were designed using AutoCAD and printed out as masks on the film (CAD/Art Services Inc.). The replica molds were made of SU-8 2050 negative photoresist (MicroChem Inc.) spun on silicon wafers at a speed of 2000 RPM and UV-exposed under masks using a Karl-Suss MJB aligner to achieve pillars with a height of 60 μm. PDMS membranes with a height of approximately 40 μm were obtained by 9 : 1 mixing of the base agent and the curing agent Sylgard™ 184 (World Precision Instruments Inc.). The 9 : 1 mixture provided higher biological compatibility than the conventional ratio of 10 : 1.¹⁷ To achieve through holes for microwell formation, 10% xylene was added to the mixture to reduce viscosity. The membranes with the height of approximately 40 μm were obtained by spinning the mixture on the mold at a speed of 3000 RPM for 1 min. The PDMS membranes were cured at 125 °C for maximum crosslinking and immersed consecutively in three solvents (triethylamine, ethyl acetate, and acetone, each for 2 h) to extract short chain oligomers. The membranes were baked in a vacuum oven at 137 °C overnight to remove the solvents. A solution of 70% ethanol was sprayed on the underside of the membranes as a lubricant, which allowed the membranes to be quickly slid and aligned with the lines of MEA electrodes under microscope. Finally, the assembled biochips were baked at 50 °C for 2 h to create a permanent bond.

The original MEA surface and the overlying PDMS membrane were not natively supportive of cell attachment and spreading. Therefore, a series of surface-modification treatments were conducted to improve the biocompatibility of the substrate, such as transforming the siliconitride (MEA insulating layer), indium tin oxide (ITO, electrode material), and PDMS (elastomeric membrane) substrate into a cytophilic surface for promoting cell attachment and spreading. The biochips were supersonically cleaned in 100% ethanol and activated by oxygen plasma for 10 min at 150 mTorr using the “high” setting on a Harrick Plasma

PDC-32G cleaner/sterilizer. Immediately following the plasma treatment, the biochips were immersed in a solution of fibronectin-DPBS ($100 \mu\text{g mL}^{-1}$) and placed in an incubator overnight at 37°C with $5\% \text{CO}_2$. On the following day, surfaces were rinsed three times with DPBS for 5 min each. Before cell seeding, the biochips were placed under UV light for 15 min for the final sterilization. To clean and reuse the biochips, the culture medium was replaced with 70% ethanol to kill the cells and then rinsed in DI water. The biochips were supersonically cleaned in a 1% Tergazyme™ solution for 30 min and remained in the solution overnight at room temperature to break down the tissue. After the Tergazyme™ solution was washed out, the biochips were then supersonically cleaned in DI water for 30 min and remained in DI water overnight to remove residual chemicals. Finally, the biochips were surface modified as described above for new chip preparation, and each biochip was reused up to 20 times.

Cell bridge formation

Freshly isolated cardiomyocytes were seeded on the biochips at a concentration of $2 \times 10^6/\text{mL}$. After four days the cardiac muscle fibers formed and started contraction within the long microwells. Micropipettes fabricated typically for patch clamp experiments using Brown-Flaming puller (*P-97*, Sutter Instrument Co.) were used; the tips were polished using a microforge for a smooth edge. A prepared micropipette was mounted onto a motorized three-dimensional micromanipulator and connected to a 20 mL syringe with a plastic tube. A middle portion of the muscle fiber in each microwell was removed by strong suction under the inverted microscope; the small gap thus created averaged $120 \mu\text{m}$ in length. After ensuring that neither cells nor cell debris was present in the gap, the biochip was sealed into the laser-deposition chamber, and individual cells, including rMSCs and control cells (cardiomyocytes and fibroblasts) were laser-patterned into the gap to bridge the separated cardiac muscle fibers. Since different cell types experience different optical forces and thus display different moving speed, although our cardiomyocyte population might have 5% cardiac fibroblasts, only cardiomyocyte were selected by the laser guidance cell patterning procedure.

The laser source was a tunable Ti-sapphire laser (Spectra-Physics 3900S CW) operated in the TEM_{00} mode with a wavelength of 800 nm. To generate sufficient optical force for cell patterning, the laser beam was expanded by a pair of lens ($f = 15 \text{ mm}$ and $f = 20 \text{ mm}$) and focused into the cell deposition chamber using a long-working-distance objective (EPI L Plan Apo 20X, $\text{NA} = 0.35$). The details of the laser-patterning system were reported in our previous publication.¹⁸

For a typical laser-patterning procedure, the system was initialized by issuing parameters to the laser, imaging system, motorized stage, and microinjection pump to enable them “ready for operation.” Stem-cell suspension at a concentration of $30,000 \text{ cells mL}^{-1}$ was loaded into a $50 \mu\text{L}$ microsyringe coupled with a hollow fiber, which was sealed in the laser-deposition chamber above the MEA chip. The cell-feed ratio was optimized for single-cell flow by adjusting the volume (50 nL) and speed (25 nL s^{-1}) per injection. The injection of cells into the chamber triggered the motorized stage to bring those cells into the field of view. Then the cell was maneuvered to the center of the monitor screen and the laser shutter was opened to capture the cell. Once the cell was captured by the laser beam, the user carried the cell along in the laser-guidance region to the gap in the cardiac muscle fiber with transverse and vertical manipulation speeds of $100 \mu\text{m s}^{-1}$ and $25 \mu\text{m s}^{-1}$, respectively. During typical laser patterning, the power of the laser beam was set to 150 mW inside the chamber, which caused no cell damage and provided excellent radial and axial forces for manipulation. When the laser focus and the captured cell came within $100 \mu\text{m}$ above the MEA surface, laser power was automatically reduced to 50 mW to avoid overheating the electrodes (otherwise the high temperature around the electrode would generate a convection force that

would push the cell out of the guidance region). The whole laser-patterning process (10–30 s) was repeated until the gap was filled with stem cells as shown in Fig. 2(a). About twelve hours after laser-patterning (Day 1), the stem cells spread and formed cell bridges connected to the adjacent cardiomyocytes as shown in Fig. 2(b).

Electrical conduction analysis

The MEA chips were placed in the holder of a commercial data acquisition system (MultiChannel System Inc.). A heating element underneath the device regulated the temperature at 37 °C. MCS Rack V3.5.5 data acquisition software was used with A/D conversion set at a 50kHz sampling rate. The raw signals from all the electrodes were simultaneously recorded. Local activation time (LAT) was determined as the time with maximal upstroke velocities ($-dV/dt_{\max}$) of the electrogram recorded on each electrode. The local conduction velocity (LCV) was calculated by dividing the distance between two adjacent electrodes (200 μm) by the interval between their LATs. The electrical propagation along the cardiac muscle fiber with a cell bridge was assessed daily at twelve hours after laser-patterning (Day 1) up to Day 7.

Immunocytochemistry

On Day 5, immunocytochemistry was performed on the cardiac muscle fibers with stem-cell bridges to visualize muscle structure and junction protein in detail. To protect the MEA chip for reuse, the immunocytochemistry was conducted with an elastomeric membrane attached to a $22 \times 22 \text{ mm}^2$ glass coverslip without an MEA. The microscale features of the membranes were exactly the same as the ones used for the MEA chip. The cardiac muscle fibers and stem-cell bridges were prepared by the same procedure as those for the MEA chip. The cells were fixated, permeabilized, and blocked in 4% paraformaldehyde (10 min), 0.1% Triton X-100 (15 min), and 2% bovine serum albumin (BSA) with 4% donkey serum, respectively. Next, the cells were labeled with the primary antibody (mouse anti-sarcomeric α -actinin (1 : 400), rabbit anti-connexin 43 (1 : 200)) at 4 °C overnight. Excess primary antibody was removed by a triple wash in PBS, and cells were stained with secondary antibodies (Cy3-conjugated anti-mouse IgG, Cy3-conjugated anti-rabbit, 1 : 200) at room temperature for 2 h. After three washes with PBS, the slides were prepared with ProLong® antifade kit mounting medium (Invitrogen Inc.) and observed under a fluorescent microscope.

Gap junction assessment

To determine the role played by gap junctions in electrical conduction through the stem-cell bridges, carbenoxolone (Sigma-Aldrich), a reversible gap junction uncoupler, was dissolved in DPBS at various concentrations (50 μM –500 μM) to block the gap junctions within the cardiac muscle fibers. On Day 7, carbenoxolone was applied to the cardiac muscle fibers with either cardiomyocyte bridges or stem-cell bridges for 15 min. Cell-bridge models with the addition of only culture media were used as corresponding controls. The effect of a specific carbenoxolone concentration on electrical conduction was studied by monitoring conduction velocities. Then, the cultures were washed twice by DPBS and incubated with culture medium for 30 min. Conduction velocities were measured again to test whether conductivity was restored.

Results

Cardiac muscle fiber with a cell bridge

On the third day after the cell bridge was created, the muscle fiber bridged by stem cells was observed synchronously beating in the microwell. The cardiac muscle fiber was stained by sarcomeric α -actinin (green), and the stem-cell bridge in the middle was tracked by DiI (red)

as shown in Fig. 3(a). The cardiac muscle fiber exhibited an aligned and elongated morphology inside the long microwell as shown in Fig. 3(b), which mimicked the *in vivo* structure of heart tissue. Stem cells formed a bridge to connect the separated muscle fibers as shown in Fig. 3(c). The connexin 43 staining indicated that connexin 43 was locally concentrated at the connection areas between stem-cell bridges and cardiac muscle fibers as shown in Fig. 3(d); this result suggested that gap junctions might form and serve as electrical propagation channels through the bridges. A three-dimensional confocal image of the connection area between the muscle fibers and the cell bridge showed that the cardiac muscle fiber grew higher than 30 μm by accumulating multiple layers of cardiomyocytes inside the microwell (figures not provided). However, only a thin layer of stem-cell bridge grew on the bottom of the microwell because the laser-patterning technique can precisely control the cell number to be just enough for cell-bridge formation.

Extracellular field potentials

Typical extracellular electrograms, shown in Fig. 4, were recorded on the second day after cell-bridge formation (Day 1) from a cardiac muscle fiber with a stem-cell bridge. The field potential recorded from the electrode near the stem-cell bridge was much smaller than the one recorded from the adjacent electrode under the cardiac muscle fiber, and the pulse widths in these two electrograms can be identified through their waveforms. The electrical properties of different cell bridges on Day 1 and Day 7 are compared in Table 1. The amplitudes (FPA) and maximal upstroke velocities (MUV, dV/dt_{max}) of extracellular field potentials of cardiomyocyte bridges (positive controls) were significantly higher than that of stem cells and fibroblasts (negative controls), which indicated that more ions were exchanged due to action-potential formation locally in the cardiomyocyte region than in the stem cell or fibroblast regions. Although the FPA did not significantly differ between stem cell bridges and fibroblast bridges on Day 1, the difference became significant on Day 7. This suggested that the electrical properties of the bridged stem cells were regulated by the muscle fiber from fibroblast-like to cardiomyocyte-like. There was no significant difference in the field potential duration (FPD) among these three types of bridges on Day 1; on Day 7, only the cardiomyocyte bridges showed significant FPD reduction. The reduction in FPD might be the result of the expression of sodium channels. Our unpublished data with single-cell RT PCR demonstrated that expression of sodium channels was absent on the cell membranes of rMSCs that were cocultured with cardiomyocytes but not in contact with the cardiomyocytes. At Day 5, however, sodium channels were weakly expressed on rMSCs that had contacted with cocultured cardiomyocytes.

Electrical conduction velocities

The LCVs of a bridged muscle fiber were calculated using the recorded extracellular field potentials from Day 1 to Day 7. First, the effect of cardiomyocyte alignment on the LCVs was assessed by comparing two types of cardiac muscle fibers of different microwell width. A biochip was created with long microwells of 50 μm in width; at this width a cardiac muscle fiber with fully aligned cardiomyocytes cannot be produced. The LCVs of cardiac muscle fibers in this 50- μm biochip were lower than that of the 30- μm biochip on Day 7 as shown in Fig. 5(a), which indicated that the higher LCVs were related to cell alignment.

Stem-cell bridges, shown in Fig. 5(b), initially had much lower LCVs than those of the cardiac fiber areas, but these gradually increased with days of culture and reached a stable level on Day 5. The cardiomyocyte bridge, shown in Fig. 5(c), integrated with the host cardiac muscle fibers in the first twelve hours and restored their conductivity, making it equal to the cardiac fiber areas. This suggested that cardiomyocyte bridges can rapidly respond to the host cardiac muscle fiber and recover the electrical conduction of the broken fiber. The LCVs of fibroblast bridges, shown in Fig. 5(d), increased in the first three days

and dropped in the following four days. A comparison of the LCVs among three types of bridges is shown in Fig. 5(e). The stem cells exhibited higher and more stable conduction velocities than the fibroblasts, which indicated that the stem cells had higher electrical compatibility with native cardiac muscle fibers. The fibroblast bridges showed a small potential to propagate the electrical signals along the cardiac muscle fiber but failed to maintain a stable electrical conduction after their proliferation, which is comparable to scar formation in the *in vivo* studies.

To test whether the conduction activities recorded from our cell bridge models were mediated through gap junctions, an important functional cardiac muscle structure, carbenoxolone was added to block the gap junctions within cardiac muscle-fiber models with either cardiomyocyte bridges or stem-cell bridges at Day 7. The LCVs of the stem-cell bridges were significantly reduced at carbenoxolone concentrations higher than 150 μM and fully abolished at concentrations higher than 500 μM as shown in Fig. 5(f). The LCVs of the cardiomyocyte bridges were reduced at concentrations higher than 200 μM , and the conduction was approximately 10 cm s^{-1} at a concentration of 500 μM . The high carbenoxolone concentration that was required to block electrical propagation indicates that the density of gap junctions inside the cardiomyocyte bridge was higher than that inside the stem-cell bridge. LCVs of both bridge models returned to their original levels after they were rinsed for 30 min. Simultaneously, synchronization of each cardiac muscle fiber was restored. These findings indicated that gap junctions controlled the myocyte-myocyte and stem cell-myocyte electrical couplings.

Discussions

In our cardiac muscle-fiber model, cardiomyocytes acquired *in vivo*-like cell morphology with highly organized striation patterns. This is vital for the development of *in vivo*-like *in vitro* models of ventricular structure and function. A similar model created by McDevitt and coworkers showed that cardiomyocytes in their fiber model developed highly aligned myofibrils with normal diameters and bipolar cell junctions with intercalated disk connections that included spatially localized N-cadherin and connexin43.¹⁰ Thomas and coworkers reported their investigation on the electrophysiological properties of aligned cardiomyocytes in cardiac muscle fibers.¹⁹ They found that conduction velocities and action potentials were faster and more similar to adult mouse myocardium in cardiomyocytes grown in strands *versus* those grown in randomly oriented cultures. In Pijnappels's studies,²⁰ the conduction velocity of randomly oriented cardiomyocytes ($17 \pm 0.2 \text{ cm s}^{-1}$) was much smaller than that of our aligned cardiomyocytes ($42 \pm 5.9 \text{ cm s}^{-1}$); these data of ours were similar to Thomas's results on aligned cardiac muscle fiber ($45 \pm 17.2 \text{ cm s}^{-1}$). As shown in Fig. 5(a), our comparison of data obtained from two cardiac muscle-fiber models, one with less aligned cardiomyocytes ($29 \pm 2.8 \text{ cm s}^{-1}$) and the other with more aligned cardiomyocytes ($42 \pm 5.9 \text{ cm s}^{-1}$), further demonstrated the effect of cell alignment. This aligned structure may also facilitate electrical conduction of stem-cell bridges, which exhibited a higher conduction velocity ($25 \pm 5.7 \text{ cm s}^{-1}$) in our research than that reported by Pijnappels ($7 \pm 1 \text{ cm s}^{-1}$). The length of cell bridges differed between our model ($\approx 120 \mu\text{m}$) and Pijnappels's (250–350 μm), which may also be responsible for the higher conduction velocities obtained from our stem-cell-bridge model.

One of the early cell-based cardiac repair therapies was established with the transplantation of autologous skeletal myoblasts that normally mediate regeneration of skeletal muscle.²¹ It has been shown that mature skeletal muscle cells do not express the gap-junction proteins required for electrically coupling of cells,²² and available physiological data suggest that these autologous grafts do not beat in synchrony with the rest of the heart.²³ These reports demonstrate the importance of gap-junction formation between transplanted cells and host

cardiomyocytes. In our cardiac muscle model, a gap junction uncoupler, carbenoxolone, significantly reduced the conduction velocity of entire muscle fibers with stem cell bridges. In addition, with the removal of the blockers, the originally established electrical propagations were resumed. These data indicated that electrical propagation in our model relied on gap-junction formation within the muscle fiber and suggested the potential for stem cells to form gap junctions with host cardiomyocytes during cellular therapy. Our connexin 43 staining showed that gap junctions concentrated at the connection area between the cardiac muscle fiber and the stem-cell bridge, but relatively low expression was found inside the bridge between the stem cells. Therefore, longer bridges or higher cell numbers may obstruct the electrical propagation across the bridge. The stem-cell bridges created by the laser-patterning technique used a defined number of cells, only enough to cover the gap, as opposed to conventional methods in which the stem cell bridges were formed by multiple layers of cells with a largely unknown number of cells. This uniqueness will facilitate future detailed studies on the dependence of conductivity on bridge length and cell numbers.

Fibroblasts are the dominant population among cardiac nonmyocytes. They are arranged in sheets and strands that run parallel to the prevailing direction of muscle fibers between the layers of myocardium. In previous studies, fibroblasts of cardiac origin were found to be capable of electrically coupling to cardiomyocytes and to activate cardiac tissue over a distance up to 300 μm .²⁴ However, there were conflicting conclusions on electrical coupling between fibroblasts and cardiomyocytes among different authors. Kohl²⁵ observed electrotonic interaction between cardiomyocytes and fibroblasts, but Beers and coworkers found that fibroblasts failed to conduct electrical signals between cardiomyocytes.³ According to our results, fibroblasts had limited capability to electrically couple with cardiomyocytes, especially when the fibroblasts proliferated inside a cardiac muscle fiber after three days of culturing. In cardiac diseases, such as myocardial infarction, collagen deposition by fibroblasts increases dramatically.²⁶ The associated fibrous tissue acts as an insulator.²⁷ This formation of fibrous tissue may produce passive obstacles for conduction, thereby increasing cardiac electrical heterogeneity and contributing to arrhythmogenesis. Our fibroblast-bridge model simulated this phenomenon of fibroblast electrical coupling.

Mesenchymal stem cells (MSCs), the stem cell source used in our research, have been investigated *in vitro* for cardiac repair, and promising results were obtained on their transdifferentiation into the cardiomyocyte phenotype.²⁸ In an *in vivo* study, however, Martin and his coworkers²⁹ directly injected MSCs into infarcted pig hearts and found that although the MSCs expressed several muscle markers, their morphology resembled fibroblasts more than cardiomyocytes. In our study, we compared the electrical conductivity of stem cells and fibroblasts in an *in vivo*-like cardiac muscle model to determine their ability to electrically couple with native cardiomyocytes. The different electrical conductivities of stem cells and fibroblasts were not only exhibited by their conduction velocities but also by their ability to maintain continuous conduction in long-term culture. Although the conduction velocities of stem-cell bridges did not exhibit significant change during the first two days, they gradually increased in the following three days to a stable value. This suggested that stem cells may interact with adjacent cardiomyocytes and be regulated to become more electrically compatible.

Conclusions

In conclusion, using laser-patterning and microfabrication techniques, we developed a cardiac muscle model with multiple muscle fibers with cell bridges on MEA chips, which allowed on-chip analyses of electrical conductivities of different cell types on different culture days. The aligned morphology of cardiomyocytes in the cardiac muscle fibers mimicked *in vivo* tissue structure. The cell number for bridge formation could be precisely

controlled using the laser-patterning technique, which provided an identical cellular condition for systematic comparison among different donor cells. Stem-cell bridges showed a strong ability to conduct electrical signals along a cardiac muscle fiber through gap junctions; thus they can be considered a promising cell source for designing efficient cardiac cell therapies.

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Fig. 1. (a) The microwells on the elastomeric membrane were aligned with the MEA electrodes; (b) stem cells (pink spheres) were laser-patterned to form a bridge connecting two separated cardiac muscle fibers (green) inside one microwell. Optical force was used to trap a cell to the axis of the laser beam and guide it downward to the MEA surface.

Fig. 2. Stem cell bridge (a) immediately after laser-patterning and (b) 12 h later. Red: DiI for stem-cell tracking.

Fig. 3.
 (a) Cardiac muscle fiber with a stem cell bridge; (b) cardiomyocytes exhibited aligned morphology; (c) stem cells were well connected to the adjacent cardiomyocytes; and (d) gap junctions (white arrow) expressed between the cardiac muscle fiber and the cell bridge.

Fig. 4. Typical electrograms recorded from two adjacent electrodes, one from a stem-cell bridge and one from a cardiac fiber.

Fig. 5.

The LCVs of cardiac muscle fibers with (a) different width, (b) a stem cell bridge, (c) a cardiomyocyte bridge, and (d) a fibroblast bridge. (e) The conductivity of different cell bridges was compared on different culture days. (f) The effect of a gap junction was assessed using a gap junction uncoupler.

Table 1

Electrical properties of different cell bridges on Day 1 and Day 7^a

	Day 1			Day 7		
	FPA (μ V)	MUV (mV s^{-1})	FPD (ms)	FPA (μ V)	MUV (mV s^{-1})	FPD (ms)
Cardiomyocytes	131 $\pm$ 56.1	4.4 $\pm$ 1.86	98 $\pm$ 24.1	551 $\pm$ 156	27.6 $\pm$ 7.8	63 $\pm$ 14.4
Fibroblasts	33 $\pm$ 13.4	0.8 $\pm$ 0.41	115 $\pm$ 18.4	47 $\pm$ 8.4	1.2 $\pm$ 0.25	104 $\pm$ 22.1
Stem cells	40 $\pm$ 12.2	1.2 $\pm$ 0.38	103 $\pm$ 17.9	143 $\pm$ 32.2	4.1 $\pm$ 1.1	92 $\pm$ 21.8

^aFPA: field potential amplitude; MUV: maximal upstroke velocity; FPD: field potential duration.